M.TECH THESIS

On

ANTIMICROBIAL ACTIVITY OF SULPHUR NANOPARTICLES ON DANDRUFF CAUSING *MALASSEZIA* YEASTS

Submitted by

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CERTIFICATE

*This is to certify that the project on "***Antimicrobial activity of sulphur nanoparticles on dandruff causing *Malassezia* yeast",** *has been completed by Ms Sreerupa Sarkar, Roll no.-210CH1044, of M.Tech (Regular), under my supervision as a part of her final year project.*

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Last but not the least I thank my friends for being a strong and genuine support throughout my endeavours, to the best of their abilities.

Thanking you,

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ABSTRACT

With dandruff being a common everyday problem and the market loaded with antidandruff shampoos and such skin care products, it is obvious to assume resourceful research into this area would be both objective to present scenario and lucrative potentially. Nanoparticles are frequently in use in some very powerful antimicrobial, antifungal cosmetics nowadays especially silver. However most metallic nanoparticles are harsh and often toxic, and concern remains on their ill effects. Sulphur, certified as biocompatible to eukaryotes and as soil nutrient is often used in medicine. Though sulphur nanoparticles are not much worked with it still is a strong antifungal agent and have been used in macro amount in shampoos. Our work concentrates on synthesizing sulphur nanoparticles in a surfactant system, insitu, such that it could possibly be used in curbing dandruff naturally. A surfactant has some natural antimicrobial property and therefore such combination could be a potential antidandruff hair washing formulation. To check its antidandruff activity, experiments have been conducted on *Malassezia furfur* the causal organism for seborrheaic dermatitis or dandruff, which have been cultured for such study in our lab. Spectroscopy based microbial growth kinetics and colony inhibition studies have been performed to show that nanoparticles of sulphur reduce proliferation of *Malassezia* yeast colonies abundantly, and cause cellular damage which inhibit its growth and viability considerably.

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

"The smallest of things might have the deadliest of impacts", is a hard learned truth mankind has learned through long years of suffering and surviving. Entities only a few microns of size wiped out entire populations, in form of small pox, and plague and other diseases in one fatal blow. Ironical as it might be, Europe had its population reduced to one third by the 'Black Death', in the Middle Ages, a casualty far more subtle and bigger than what it suffered in the world wars. Though antibiotics and other therapies have been very useful in fighting them, much like H.G.Wells classic idea in 'War of the Worlds', the inevitable weakness of our defences still stands farce in face of disease.

After the 1950s, with the boom in genetics and biotechnology, and a general impetus to hygiene consciousness, life expectancy suddenly crossed 100years on an average and most known pathogenic diseases were deemed curable. However new virus-influenced physiological syndromes and nonpathogenic disorders now became statistically significant and a focus of medical science. Today, 'Cancer' and 'AIDS' evokes the same dreaded feeling as 'plague' and 'leprosy' would elicit in the middle ages.

As they say, 'Theres always a bigger fish'. But here 'big' refers more to power than to size. To combat 'micro', we have 'nano', a new and increasingly broadening concept which is bringing a new technological and medical revolution. 'Nano', means small enough to be about 10^{-9} metres dimensionally. It refers to particles or constructs of nanometer dimensions. Notworthy it is that nanotechnology sprang out of electronics and the drive already existent from the 1900s to miniaturize gadgets and information technology. But soon it swept across all fields and began to have a heavy thrust on biotechnology and medicine.

1.1. NANOTECHNOLOGY AND NANOPARTICLES

Nanotechnology is a multidisciplinary subject dealing with the synthesis, properties, and applications of the macromolecules or particles at the nano range (10^{-9}m).

1.1.1. HISTORICAL BACKGROUND

The publication of the book, *Engines of Creation*, in 1986 was the first concrete conceptualization of the essence of nanotechnology with growing public awareness and controversy in the early 2000s, and government funds being focused on this new potential arena. But the seeds were laid much earlier when Richard Feynman, gave his speech in Caltech, for American Physical Society on December 29, 1959, describing how if very small particles could be manipulated, atomic and molecular interactions would become prominently visible and impact of gravity would be completely superceded by small range forces like Van der Waals interaction and surface tension.

But the ideas gained ground after they were used by K. Eric Drexler in the, *Engines of Creation: The Coming Era of Nanotechnology*, which took the Feynman concept in a cover article headlined "Nanotechnology" and soon the term became a sensation [Toumey, 2008].

However the first to coin the term nanotechnology came from Japanese scientist Norio Taniguchi of the Tokyo University of Science in a 1974 conference, while describing semiconductor processes such as thin film deposition and ion beam milling where characteristic control on the order of a nanometer was needed. He defined the term as- "'Nano-technology' mainly consists of the processing of, separation, consolidation, and deformation of materials by one atom or one molecule."

The first observations and size analysis of nanoparticles had been made by Richard Adolf Zsigmondy, for which he won the 1925 Nobel Prize in Chemistry. He made a detailed study of gold sols and other nanomaterials with sizes down to 10^{-9} nm using an ultramicroscope which could visualize particles much smaller than the light wavelength. In another development, the synthesis of semiconductor nanocrystals was made possible reproducibly, and its properties were studied. This led to accelerated investigations on semiconductor nanoparticles and quantum dots. With the discovery of fullerenes and scanning tunneling microscope, study and visualization of the particles were made possible. The field of interfacial and colloidal sciences, which had been studied for a long time, became the foundation of understanding nanoparticles behavior and properties. And since even for the same element, the physical and chemical properties of nanosized particles were remarkably different from the bulk particles, soon the study of nanomaterials and nanotechnology became a vast and discrete field of knowledge by itself.

The early 2000s saw the high throughput of nanotechnology in commercial products, although most applications were limited to the bulk use of passive nanomaterials. Examples include disinfectants and household appliances such as 'Silver Nano', TiO_2 and ZnO nanoparticles in sunscreen, silver nanoparticles in food packaging, clothing, cosmetics and some food products, carbon nanotubes for stain-resistant textiles, and cerium oxide as a fuel catalyst. With all this we come to the age of the nano-technological revolution.

1.1.2. NOMENCLATURE

Much of the work being done today that bears the tag name 'nanotechnology' is not nanotechnology in the original meaning of the word. Nanotechnology, in its traditional sense, means building things from the bottom up, with atomic precision, a theoretical as envisioned by Feynman. With time and prospects, the definition of nanotechnology got modified and altered.
The term 'nano' stands as a prefix to a measurable quantity in space or time, and originates from the Greek word 'nanos' meaning dwarf.
. The NNI (National Nanotechnology Initiative) defines- 'The branch of technology that deals with dimensions and tolerances of less than 100 nanometers, especially the manipulation of individual atoms and molecules is termed as nanotechnology and nanomaterials as 'one having at least one dimension in nanorange'. A simpler definition states nanomaterials as materials which have at least one dimension of less than 10^{-7} m (or 100nm) [Kahn, 2006].
The importance of size, in nanomaterials is immense, because unlike in bulk materials, all properties of nanosized materials change with their size. Quantum confinement, surface plasmon resonance, supraparamagnetism and phonon resonance are some characteristic features of certain nanoparticles, which are exclusive for the material only due to their nano size (i.e. the features are not shown in bulk state) and vary in correspondence to small variations in the size. Due to these features, nanoparticles and materials are so sought after in the scientific arena.

1.2 TYPES OF NANOPARTICLES

The smallest particles considered a nanoparticle contain tens or hundreds of atoms, with dimensions at the scale of nanometers, hence nanoparticles. Nanoparticles can be categorized in

several ways. Morphologically they can be nanoclusters, nanopowders, or nanocrystals. Nanoclusters are as semi-crystalline structures with at least one dimension between 1-10 nm and narrow size distribution. A nanopowder by definition is "an agglomeration of noncrystalline nanostructural subunits with at least one dimension less than 100nm." Nanocrystals are single crystalline homogenous units. Nanocrystals are nanomaterials crystals with at least one dimension less than 100nm. Those are just the gross sectors of nanoparticles.

Structurally, according to design, nanoparticles can be solid, homogenous particle, layered core-shell heterogenous particle or hollow particles. Generally advanced synthetic nanoparticles are manufactured using several types of elements or compounds in strata and in 3D would assume the form of core/shell structure. Hollow particles are also much in demand because they can encapsulate other molecules within them.

Besides chemically nanoparticles can be metals, generally pure noble metals, iron, cadmium, zinc, titanium and their compounds, or nonmetals like silicon, sulphur and so on. Composite molecules, including organic, polymeric, inorganic materials are more advanced nanomaterials which are being developed suiting to their objective.

1.3 FUNGAL DISEASES

Fungal diseases are very common but mostly not as fatal and fearsome as bacterial or viral one, so often getting unattended to. In fact, fungal studies lead to the discovery of the first antibiotic, Penicillin, derived from *Penicillium notatatum*. But in plants fungal diseases are quite important. A variety of diseases like 'blights', 'rusts', 'smuts', 'mildews', 'scabs' are fungal diseases. Hence indirectly fungal infections are very important to us, from perspective of agriculture and horticulture, also in fishery sciences and poultry.

In human or higher animals, fungus diseases are mostly mentioned in relation to infections of superficial and subcutaneous organs especially skin and other peripheral organs. Only in rare and severe cases, fungal infections of lungs (due accidental inhalation of fungal spores), oesophagus, bronchi and sometimes male reproductive glands have been reported Some common fungal diseases being ringworm, pityriasis versicolour, common dandruff, severe seborrheic dermatitis.

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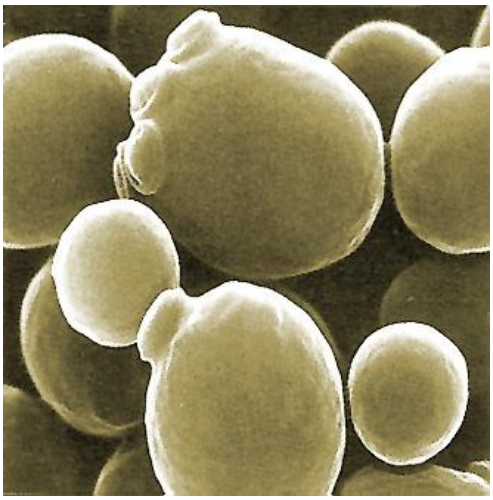
(b) 

Figure 1: Two industrially important fungi: (a) *Aspergillus niger,* (b) *Saccharomyces cerevisae* (electron microscope image).

Fungal toxins known as mycotoxins are sometimes cause severe dieases like carcinogenic aflatoxin from *Aspergillus flavus*. Others like yeast *Saccharomyces cerevisae*, and *Aspergillus niger*, are widely known for Beer and wine industries.

CHAPTER 2

REVIEW OF LITERATURE

2 REVIEW OF LITERATURE

Though not much work has been done on nanoaparticles developed for cosmetic cures, various related studies have been done in this field since 1980s. We therefore move into the background study of our work.

2.1 APPLICATION OF NANOPARTICLES

Researchers have been developing a vast array of products based on nanoparticles, some of which open to the market for public consumption. Nanotechnology has miraculous effects on housecleaning products, the nanoparticles comprising the cleaning fluids have been engineered to absorb dust and grime in contact. To a layman it would be like to spray the dirt and watch the nanoparticles make it magically disappear! Another consumer wizard is the self-cleaning fabrics. In this case the nanoparticles have been similarly engineered to “eat” stains, while another approach is to use nano-hairs as a thin, indiscernible deposit over the fabric itself so that blemishes cannot penetrate. In both cases, extremely stain-resistant fabric, virtually impossible to soil, was developed using nanoparticles. Most of the successful attempts have been with wool and silk, but other fabrics like cotton are being stain-proofed daily [Ballauff and Lu, 2007, Montazer et al, 2012, Wu and Long, 2012].

Nanoparticles have a huge effect on electronics as it was due impetus from this area that ushered in the nanotechnology drives. Lithium nanoparticles batteries [Ma et al, 2009, Dimitrijevic et al., 2012], low temperature operating solar cells of semiconductor nanoparticles [Kanmani and Ramachandran, 2012], zinc oxide nanoparticles based UV resistant covering of wood, textiles[Farouk et al., 2010], strengthened tennis racquets of silica nanocrystal reinforcements are several other diverse applications of nanoparticles.

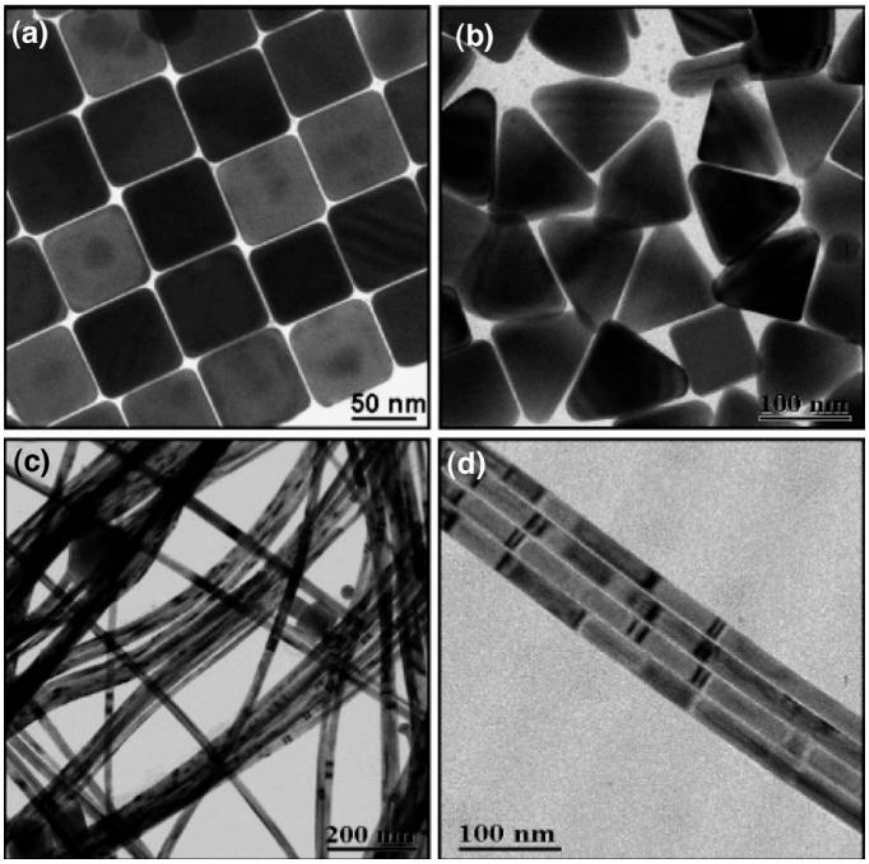


Figure 2: Silver nanoparticles of different shapes under electron microscope: (a) cubes, (b) triangles, (c) wires, (d) an alignment of wires. [Sharma et al., 2009]

But perhaps the maximum popularity of nanoparticles lies due to in their medical applications. They make them prime candidates for the fight against various unwelcome invaders of the human body because of their pathogen-sized proportions; they can be injected intravenously and are highly toxic to microbes. In fact smaller the size, higher the toxicity. It seems likely that nanoparticles are also key players in the fight against cancer as well which is often non-pathogenic in nature [Tanaka et al., 2008]. Our current cancer treatments involve traumatic and excruciating procedures , while at the same time providing unpredictable success rates of cure, especially when it comes to chemotherapy. And often it becomes a painful and costly failure, due to its mass destruction of cells tumourous and normal in general.

But nanoparticles can be adapted with sensor and photographic capabilities as well as equipped with specific drugs in encapsulated form. They would then be able to circulate through the bloodstream, using their specificity and control mechanism, locate the exact site of growth of

cancer. Modern technology boost nanoparticles with fluorescent markers or magnetic oxide/ noble metal cores so that they can be located on both optical imaging devices and MRI o even others like PET, CT etc. [Tran et al., 2010, Wang et al., 2008].

Some properties of nanoparticles for which they find huge scope of applications include:

1) Optical: Scientists have engineered nanoparticles for anti-reflection product coatings, producing a refractive index for diverse surfaces, and also providing optical/optoelectric sensors for use in diagnosing cancer [Janib et al., 2010, Zhang et al., 2010].

2) Magnetic: Nanoparticles can potentially increase the density of various storage media, and supraparmagnetic cored particles like ferrites, used as MRI constrast agents as previously alluded to [Choi et al., 2010, Gerion et al., 2007].

3) Thermal: Precisely engineered particles could recover heat transfer of solar energy from collectors to their storage tanks. They could also enhance the cooling process currently used by transformers in these types of processes [Kalaiselvam et al., 2012].

4) Mechanical: Nanoparticles could provide improved shear resistance for almost any mechanical device and used as strengthening material. They could also provide anti-corrosion abilities, as well as creating entirely new composites and structural materials like carbon nanotubes that are lighter, stabler and stronger and also flexible than those we use today [Liu et al., 2011, Avila et al., 2010].

5) Electronic: Because of their tiny size, nanoparticles are the most prevalent choice to aid in the production of high routine delicate electronic gadgets, providing not only high conductivity materials, but also sleek lustrous appealing parts. And when it comes to advertising, nano-electronics pose as wonderful electricity-efficient digital displays that are less expensive to produce, brighter in color, and also bigger [Bera et al., 2010].

6) Energy: Nanoparticle batteries have been developed as longer-lasting alternatives to those we use conventionally with higher energy density, and are increasingly used in lithium batteries construct. Metal nanoclusters have potential revolutionary applications for hydrogen storage, extremely efficient electrocatalysts for fuel cells, thus paving the way for practical and renewable energy, already having established an ability to improve solar panel efficiency.

7)Biomedical. As mentioned earlier, nanoparticles have had the greatest impact on medicine and health care products. Together with the concepts of biotechnology, it has helped in

developing several revolutionary approaches to diagnosis and treatment of ailments.Nanoparticles have been used to produce "quantum dots," optically or amperometrically sensitive objects like biosensors, which can detect diseases, as well as interactive refreshments and beverages that make taste based adjustment of flavor, or in some cases alter their nutrient content based on your state of health [Sharma et al., 2006, Selvan et al., 2010, Swierczewska et al., 2010].

Soon we will discuss some details properties of nanoparticles which would have biomedical applications and medicinal use.

2.2 ANTIMICROBIAL ACTIVITY OF NANOPARTICLES

Nanoparticles, because of their size, can easily permeabilise through the cell boundaries of living cells. It is due to this reason, that cellular uptake of nanoparticles is several times more than bulk particles. It can be argued, that because of their benign nature, the cells, toxicity removal systems, like lysosomal digestion and exocytosis processes could easily remove the particles, even if they enter. But however, since the rate of accumulation of these nanoparticles, far exceed the cells ability to remove them, the cells structural and functional integrity soon crumbles, as the particles begin to interject and obstruct its physiological processes, firstly by simply 'blocking the way' and secondly by interacting randomly to biomolecules, which were meant to interact differently, for natural metabolism. Due to the extremely small size of the nanoparticles (NPs) it may interact directly with macromolecules such as DNA. Moreover nanoparticles are easily translocated to other tissues, using the circulatory system. So simply speaking, the nanoparticles are toxic to a cell because it can blend in with any structural or functional component of it, and thereby disrupting, normal cellular function. Several studies have been done to study the nature of interaction between biological components and nanoparticles, and most obviously its impact on biomembranes (like cell wall, cell membrane and nuclear membrane), since they are the first barrier to the nanoparticles entry. Disruption patterns of polycationic organic nanoparticles on model biological membranes and living cell membranes have been shown to occur even at nanomolar concentrations. The degree of disruption is related to size and charge of nanoparticle, as well as to the phase-fluid, liquid crystalline, or gel-of the biological membrane [Leroueil et al., 2007]. Not all nanoparticles produce these adverse health

effects—the toxicity of nanoparticles depends on various factors, including size, aggregation, composition, crystallinity, surface functionalization, etc. It is also very important to recognize that not all nanoparticles are toxic, some have been found to be nontoxic, and others, benefectory to the body [Derfus et al., 2004, a, b].

Table1: Nanomaterials, their morphologies, and their relative cytotoxicity index RCI on murine macrophage cells [Buzea et al., 2007].

Material	Mean aggregate size (μm)	Mean particle size (nm)	RCI (at 5 μg/ml)	RCI (at 10 μg/ml)
Ag	1	30	1.5	0.8
Ag	0.4	30	1.8	0.1
Al_2O_3	0.7	50	0.7	0.4
Fe_2O_3	0.7	50	0.9	0.1
ZrO_2	0.7	20	0.7	0.6
TiO_2 (rutile)	1	Short fibers 5–15 nm diam.	0.3	0.05
TiO_2 (anatase)	2.5	20	0.4	0.1
Si_3N_4	1	60	0.4	0.06
Asbestos Chrysotile	7	Fibers 20 nm diam., up to 500 aspect ratio	1	1
Carbon black	0.5	20	0.8	0.6
SWCNT	10	100 nm diam.	1.1	0.9
MWCNT	2	15 nm diam.	0.9	0.8

Though the toxicity nanoparticles is grave concern and a lot of effort is given to study and counter its toxic effects, the toxic nature itself serves a good purpose, when and as long as the victim is a microbial pathogen. One of the direct uses of nanoparticles in medicine is their employment in inducing destruction of these pathogens, however in a controlled fashion as not to harm the body in any way. Most often metallic nanoparticles, like gold and silver, are used in

medicated cosmetic products. Silver nanoparticles are used as antibacterial/antifungal agents in a diverse range of applications: air sanitizer sprays, face masks, wet wipes, detergent, shampoo, toothpaste, air filters, coatings of refrigerators, washing machines, food storage containers. Nanostructured titanium dioxide photocatalysts have been developed for sterilizing equipment of environmental microorganisms [The project of Emerging Nanotechnologies]. Fabric containing bamboo-charcoal nanoparticles claims antibacterial and antifungal properties and is intended for use as face mask cloth [Shintani et al., 2006].

Though studies have shown that silver in cosmetic products do have long term toxic effects, they are still widely used. Milder nanoparticles, like anionic selenium or sulphur, on the other hand, are being scrutinized as less harmful yet effective alternatives.

Several case studies and microbiology studies are there to show antimicrobial properties of nanoparticles, along with their other applications. Several bacteria have shown growth inhibition is response to nanoparticles. Antimicrobial activity of fullerenes was observed on various bacteria, like E. coli, Salmonella, and Streptococcus species [Bosi et al., 2003], so for zinc oxide particles. Silver, gold and sulphur have been found to have broad spectrum toxicity towards all microbes [Brayner, 2008]. Besides, these materials in their bulk phases, are also slightly, antimicrobial, due to either their metallic nature or in case of sulphur, its interaction with bacterial and fungal cell wall.

Fungicidal diseases, though hardly ever fatal, are quite rampant in humans, as well as in plants. So, fungicidal agents are important in the field of agricultural sciences in order to protect agricultural plants from fungal diseases like green rot and apple scab. Here sulphur particles are being widely investigated, as agricultural fungicidal agents, because sulphur being a soil nutrient, removes the chance of residual toxicity. Also on the other hand, sulphur drugs find applications as antifungal ointments to fight against skin infections.

2.3 SCALP INFECTIONS AND DANDRUFF

The common skin infections in humans or higher animals are generally due to fungal infections. Most fungal parasites are are however, generally opportunistic pathogens and are members of the normal skin flora.

Mycotic infection of the skin may be categorized into superficial and deep fungal infections. *Malassezia furfur (Pityrosporum ovale)*, a lipophilic fungus, affects the hair and causes diseases called dandruff [Ranganathan et al, 2001] and also called pityriasis versicolar, tinea circinata, seborrheic dermatitis [Rippon et al., 2000]. However recent studies show that a closely related species, *Malassezia globosa*, is the major cause of dandruff, and *M. furfur*, is a secondary pathogen which proliferates in number extensively, along with *M. globosa*, in the affected area. Dandruff is a pathological condition, causing rusty white flakes of skin to separate and fall from the scalp. Also the flakes of dandruff and peeling of skin, is observed beyond the scalp region, in the eye corners, eyelashes, eyebrows, regions in the ears and corners of nose and also near cheekbones; all courtesy to the same pathogenesis. People who suffer from dandruff have over active sebaceous glands, which make their scalp oily [Sivamani .et al, 1999]. Often though opposite to common belief, dandruff activity is higher in males, due to the effect of male hormone testosterone which is associated with increasing sebaceous gland secretions. There is reportedly no complete cure for this disease and temporary reservation is conventional treatment. *Malassezia furfur* is pleomorphic yeast like fungus. It is referred to as *Pityrosporum orbiculare* and *P. ovale* depending on the morphology of the cells.

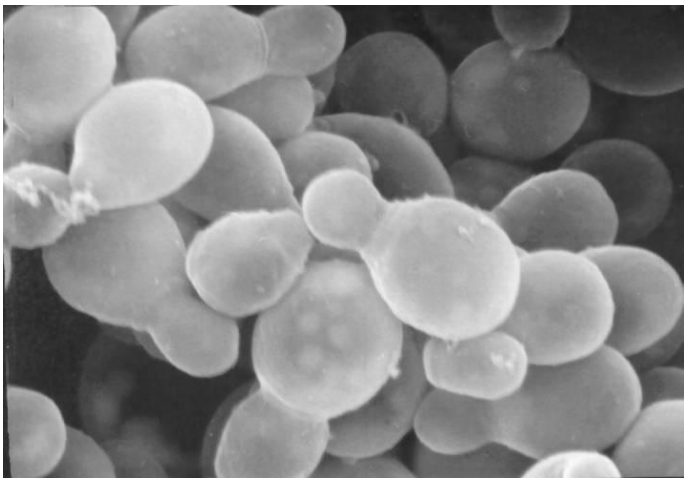


Figure 3 : SEM image of *Malassezia furfur* [Courtesy Wikipedia files].

Since dandruff is a common problem though not a serious ailment, with no permanent solution, much time, effort and money is spent by almost all of such to get rid of it. The market is loaded with antidandruff products and most are chemicals too crude for daily use. Somehow, most people associate dandruff filled hair, with lowering of their self esteem, and continue to try more

and more products having the tag of ‘antidandruff’ on it. Yet, the infection does sometimes take a severe form, with heavily affected ‘continuously peeling off’ scalp, and is known to affect other areas like eyelids, ears, corners of the nose and other regions. Seborrheic dermatitis might be hereditary. Stress, tiredness, temperature extremes, oily skin, infrequent hair washing, use of oinments with alcoholic content, or obesity might increase the risk [Sivamani .et al, 1999].

Neurologic conditions, including Parkinson's disease, and stroke is often linked with seborrheic dermatitis. Human immunodeficiency virus (HIV) has also been linked to certain cases of seborrheic dermatitis.

2.3.1 CAUSAL ORGANISMS AND CLASSIFICATION

The *Malassezia* yeasts are unique, as the only eukarotic members of the skin flora of higher animals, including human beings.

The genus *Malassezia* consist of 14 lipophilic yeasts. Its taxomy is given in Table 2.

Table 2: Scientific classification of Malassezia strains

KINGDOM	*Fungi*
PHYLLUM	*Basidiomycota*
SUBPHYLLUM	*Ustilaginomycotina*
CLASS	*Exobasidiomycetes*
ORDER	*Malasseziales*
FAMILY	*Malasseziaceae*
GENUS	*Malassezia*
SPECIES	*M.globosa*
	M. sympodialis
	M. restricta
	M. obtuse
	M. furfur
	M. slooffiae
	M. japonica

	M. nana
SPECIES contd..	*M. yamatoensis*
	M. equina
	M. caprae
	M. cuniculi
	M. pachydermatis
	M. dermatis

From the various studies conducted, it has been shown that *M. furfur, M. globosa, M. restricta* were very much opportunistic pathogens of the skin flora causing skin surface skin infections. Current data highly implicate Malassezia yeasts inthe pathogenesis of both seborrheic dermatitis and dandruff **[**Gaitanis et al., 2012**]**.

Since in this study we will concentrate on *M. furfur,* its morphology and general description is also noted.*Malassezia (*previously *Pityrosporum*) have been associated with dandruff and seborrhoeic dermatitis. This connection was theorised on circumstantial evidence that the organisms were present, in statistically significant quantities, on the skin conditions of dandruff, pityriasis versicolour and seborrhoeic dermatitis and that both responded to treatment that inhibited or destroyed *Malassezia* yeasts [Hay, 2011, Ezzi and Lynch, 2005].

The advent of sophiscated biotechnology tools such as genomic and proteomic analysis has begun to provide a new insight into the pathological mechanisms involved. Being a lipophillic yeast, Malassezia metabolises sebum components and releases oleic acid as an end product which initiates a immune-signaling pathway, attracting neutrophils and other phagocytes. This results in cytokine release, inflammatory response (hence the term ‘dermatitis’), release of ROS (Reactive Oxygen Species). This kind of immune reaction causes local epidermal cell damage, and in response to it and also triggered by excess oleic acid release, hyperproliferation of epidermal cells occur causing erroneous differentiation process and irregular keratinisation. So instead of healthy cells, degenerate cells clump and cell debris accumulate seen as flakes of dandruff.

Table 3: Population and growth of different strains on humans, based on epidemiological studies of healthy skin [Gaitanis et al., 2012**].**

No. of patients/ no. of positive cultures	**% of culture positive for**				**Location**	**Description**
	M. globosa	*M. restricta*	*M. sympodialis*	*M. furfur*		
123/107	78	1	7	21	Iran	7% mixed species correspond to avg of 8 samplings/ patient.
160/599	22	22	12	4.5	South Korea	M.globosa and M. restricta were found in all age groups commonly on scalp and M.sympodialis on the chest.
40/32	40		20	17.5	Balkan states	Healthy trunk skin of Seborrheic Dermatitis and Pityriasis Versicolour.
35/11	49	8	23	20.5	Tunis	Frequency of M. globosa was high in Pityriasis versicolour than healthy skin.
100/60	42	3	25	23	Sweden/Iran	Seborrheic dermatitis skin were significantly more colonized than atopic eczema skin.

New evidence shows the production of specific phospholipases on affected skin sites in dandruff and signalling molecules such as malassezin in seborrhoeic dermatitis [Hay et al., 2011]. Report of granulomatous dermatitis caused by M *sympodialis* first came from a clinical study on a patient and lipid was identified as a necessary substrate for growth of this genus [Desai et al., 2011].

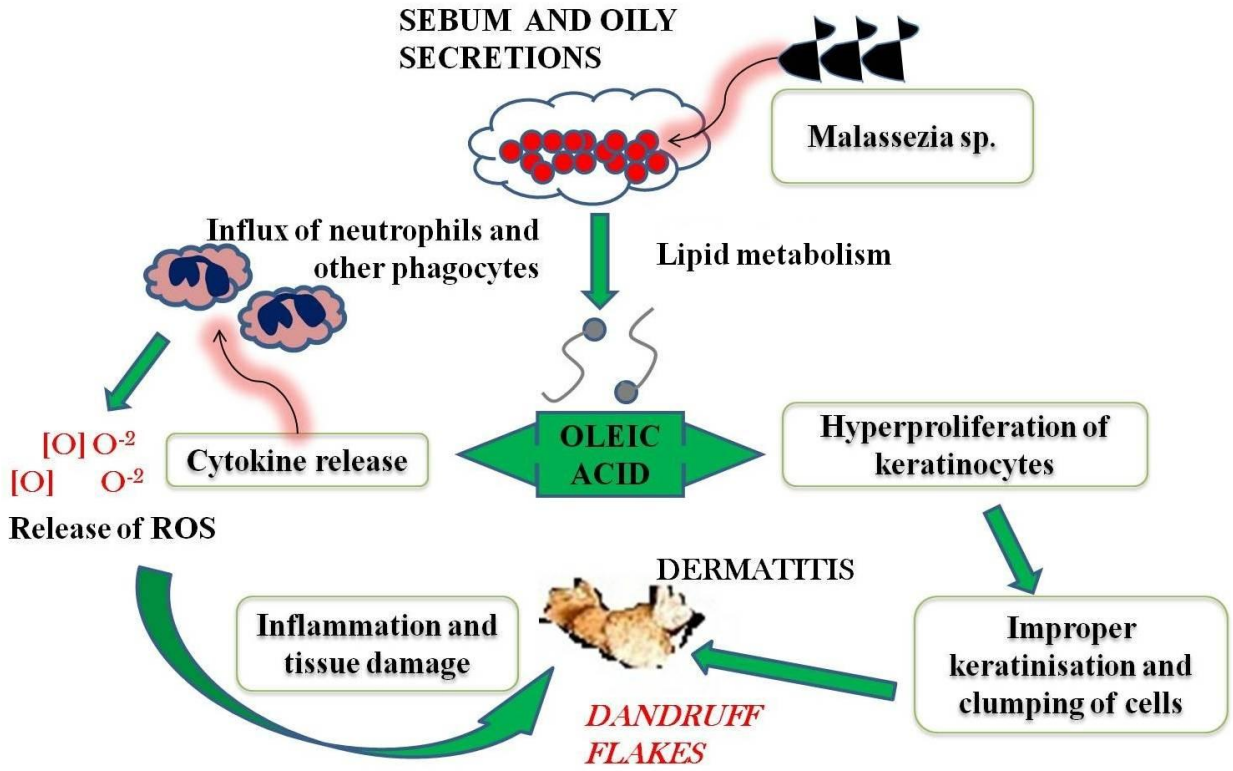


Figure 4: Pathogenesis of dermatitis

M. furfur is a cream coloured pasty yeast, with a bottle like structure, with no hyphae, or spores. Cells were reported to change shape and become rounder (i.e. elongation reduced succesively) under stressed condition, and in presence of surfactants, their outer sheath layer would thin and gradually disperse.

2.3.2 ANTIDANDRUFF AGENTS

As dandruff is common, so is so called antidandruff shampoos, and hair therapies. Shampoos use a combined formulation to control dandruff. As the pathogenesis of dandruff

involves hyper proliferation of keratinocytes, followed by deregulation of keratinization, followed by clumping together of corneocytes, manifesting itself as large flakes of loose skin, therefore, keratolytic agents such as salicylic acid and sulphur that loosen the attachments between the corneocytes and allow them to get swiped off, is a possible treatment of removing dandruff. Hence notably, sulphur is already a conventional antidandruff agent. Compound selenium sulphide is already used to treat dandruff, as it is known to have anti-Malassezia effects. [Cantoresi and Clerico, 1984, Ranganathan and Mukhopadhyay, 2010].

Other compounds include imidazole [Shuster, 1984] and hydroxypyridones [Milani et al., 2003]. Zinc pyrithione is known to inhibit growth of yeast cells of Malassezia, through inhibition of iron-sulphur proteins [Reeder et al., 2011] and is widely used in common antidandruff shampoos [Cardin et al., 1990]. On the other hand several traditional medicininal plants have been studied and their antidandruff components purified in order to promote industrial production, product efficacy and mass production. These involve Reetha (*Sapindus trifoliatus*) [Raut and Bhatia, 2008], Kalmegh (*Andrographis paniculata*) [Lava Kumar, 2009], bamboo oil (*Phyllosrachys bambusoides*) [Lee et al., 2010], *Acacia concinna* [Wuthi-udomlert, and Vallisuta, 2011].

The market is loaded with antidandruff products and most are chemicals too crude for daily use.

As mentioned earlier, nanoparticles and nanomaterials are finding immense importance and beginning to replace bulk chemicals in several antimicrobial products. Silver nanoparticles have been, tested to be used in shampoos, firstly to increase wettability and secondly to act as fungicidal agent. However potential toxic effect of silver nanoparticles in shampoos and soaps, have created quite a concern among scientists recently [Panyala et al., 2008]. While, a large number of in vitro studies indicate that Ag NPs are toxic to the mammalian cells derived from skin, liver, lung, brain, vascular system and reproductive organs and has the potential to induce genes associated with cell cycle progression, DNA damage and apoptosis in human cells at non-cytotoxic doses sulphur nanoparticles in eco-friendly and clinical trials show no alarming toxic effects on eukaryotes [Ahamed et al., 2010].A milder concoction of surfactant formulated with nanoparticles, less toxic and yet effective like sulphur,would give good wettability and be an efficient cleanser, not toxic to the skin therefore would be a lucrative direction for research.

2.4 SYNTHESIS OF NONMETALLIC NANOPARTICLES

We now concentrate on the other important aspect of our study that involves, synthesis and application of nanoparticles.

Nanoparticles or atleast simple single material nanoparticles can be synthesized in two ways, either in-situ or synthesized separately from final formulation. In case of nonmetallic nanoparticles, insitu production would require a precipitation reaction [Ghosh Choudhuri and Paria, 2012]. Generally, microemulsion and bulk aqueous system is commonly used technique in synthesizing nanoparticles in such cases, where particle size control matters most[Deshpande et al., 2008, Xie et al., 2009]. But microemulsion process involves several reagents with specific compositions, so being very cumbersome. Also other technical difficulties involve process scale up, separation and purification of the particles from the microemulsion, and consumption of huge amounts of surfactants.

The other quite simple method developed recently by Paria and co-workers is using surfactants as capping agents to control the size of particles formed. Here the nanoparticles were of a single element either metallic or nonmetallic, and the reaction was a simple reduction or disproportionate reaction respectively. The function of the surfactant was as a capping agent to control and keep the size of the particles to nanometer range. The size of the particles is a function of the nature and concentration of the surfactant and the amount of nanoparticles formed as product depended on the reactants [Choudhuri and Paria, 2010, Ghosh Choudhuri and Paria, 2012].

For sulphur nanoparticles synthesis a simple disproportionate reaction, using thiosulphate and different acids, both organic and inorganic, were used.

2.5 EFFECT OF SURFACTANTS

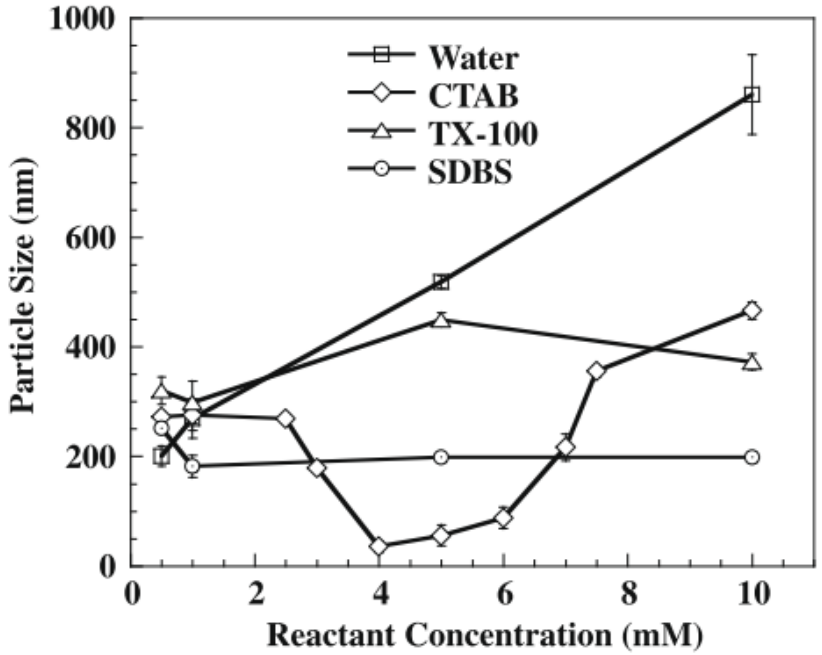


Figure 5: Variation of particle size with the thiosulphate concentration in the absence and presence of surfactants by oxalic acid catalyzed reaction [Choudhuri and Paria, 2010].

The effect of surfactants in controlling the size of the nanoparticles has been well studied by Choudhuri and Paria, in a 2011 publication. There it was noted that among the different number of surfactants used, CTAB (Cetyl trimethylammonium bromide), gives the lowest size range of particles, of about 40nm, if about 4mM thiosulphate solution is used. They also studied the distribution and stability of the sulphur particles near that concentration range and found it to be stable.

CTAB here is a cationic surfactant, which at any concentration above its CMC (Critical micellar concentration) acts as a capping agent. This means that after the elemental sulphur is formed (as product), by nucleation, the surfactant monomers get adsorbed onto the growing sulphur seeds, preventing further aggregation and expansion of the seeds into larger particles. Thus sulphur particles get restricted to nanometer range in size.

2.6 SULPHUR AND ITS BIO-INTERACTIONS

Elemental sulfur is one of the oldest fungicides and pesticides. Diluted solutions of lime sulfur made by combining calcium hydroxide with elemental sulfur in water are used as a dip for pets to destroy ringworm (fungus), mange and other dermatoses and parasites in households. Hot springs are known to heal several skin infections due to the presence of sulphur compounds and particles of low size [Lin et al., 1988].

Sulphur nanoparticles have been researched for long as antimicrobial agents, chemotherapeutic drugs, fertilizers, pharmaceuticals, rubber, fiber industries, bioleaching processes and for several other biomedical purposes [Ober, 2003]. In recent times elemental sulphur with un-quantified amounts of nanoparticles present is used in cosmetics and shampoos.

Following the mechanism explained by LaMers and coworkers [1947], a much simple in-situ synthesis protocol of sulphur nanoparticles production was developed by Chaudhuri and Paria in 2010 as mentioned earlier. Here simply an organic acid was reacted with a thiosulphate compound in presence of several surfactants, in concentrations above critical micellar concentration (CMC) which lead to slow control of the size of the sulphur found. The lowest size (30 nm) particles were obtained in a certain reactant concentration range using CTAB surfactant and oxalic acid. CTAB being a cationic surfactant with CMC 0.92mM/L should possess inherent antimicrobial property. Particles of sulphur with size distribution within 55nm can also be obtained from compounds with similar structure like CPBr (Cetyl pyridenium bromide) and CPCl (Cetyl pyridenium chloride) theoretically though experimental confirmation is absent

Lime sulphur has shown to restrict growth of *M. canis* spores [Diesel et al., 2010] and other related species. While, a large number of in vitro studies indicate that Ag NPs are toxic to the mammalian cells derived from skin, liver, lung, brain, vascular system and reproductive organs and has the potential to induce genes associated with cell cycle progression, DNA damage and apoptosis in human cells at non-cytotoxic doses sulphur nanoparticles is eco-friendly and clinical trials show no alarming toxic effects on eukaryotes [Ahamed et al., 2010,].

Also sulphur is a soil nutrient, and therefore causes no chance of soil or water pollution, if waste amounts accumulate in the environment [Palmiter and Smock, 1957, Ellis et al., 1998]. Thus it can probably be good as antidandruff, shampooing component.

2.7 CONCLUSIVE REMARKS

From all the literature available, it is inferred that nanoparticles with antimicrobial properties are much in demand. Several yeast species have been tested with sulphur, silver and other nanomaterials no work has been done conclusively to show the effect of sulphur nanoparticles on Malassezia species. Also on the other hand, several works on antidandruff agents exist but none involve sulphur particles in nano or bulk scale. The most accepted antidandruff agents are selenium sulphide, zinc pyrithione and nanosilver, the first two being sulphur compounds, however. Studies suggest that surfactants reduce microbial virility to some extent.

2.8 MOTIVATION OF PROJECT

Thus a lot of research exists on the effectivity of sulphur as fungicidal agent. Although some studies noted the potential of nanoscale sulfur against some harmful microorganisms, none reported a simple readymade one pot synthesis route and its applicability. Also the scope of sulphur nanoparticles being synthesized in the surfactant like CTAB, which is not just a detergent (therefore being a inherent washing agent), but also a fungicide and therefore the chances of this combination being a fruitful antidandruff agent is high. The reaction stoichiometry, indicates complete conversion of thiosulphate to sulphur, and the only residual component except surfactant, is oxalic acid which is biocompatible and gives a final pH of less than 3. Though Malassezia yeast grows well in a pH of 7 to 9, the final media remains at a pH of about 5-6, which the yeast can tolerate can grow normally in.

Thus by this work, we developed a cheap, and effective anti dandruff agent of sulphur nanoparticles and surfactant CTAB, utilizing a simple process, beneficial to our ultimate objective, and studied its antimicrobial properties on Malassezia yeast. This formulation would potentially reduce both synthesis, and downstream processing cost of developing an effective and non-toxic antidandruff hair washing agent.

2.9 OBJECTIVE

In this project, we aim to study the synthesis and application of sulphur nanoparticles, on dandruff causing fungus *Malassezia furfur*. The more important strain of *M. globosa* could not be worked with because of its unavailability.

The objective is to:

- Synthesize and optimize sulphur nanoparticles in surfactant CTAB
- Grow *M.furfur* in culture and optimize its growth.
- Study antimicrobial activity of sulphur nanoparticles on this strain.
- Study the morphological structure of strain before and after nanoparticles addition.

2.10 ORGANISATION OF THESIS

This thesis is contains an introduction header, which discusses about the development and concepts of nanotechnology along with its prospects, followed by a review of literature available so far on all the various aspects of the project as it combines both microbiology and nanotechnology related concepts. We would soon go into the experimental section followed by results and observations and end with a conclusive remark.

CHAPTER 3

EXPERIMENTAL WORK

3. EXPERIMENTAL WORK

3.1 MATERIALS

The surfactants CTAB (cetyl trimethyl ammonium bromide), was from Sigma Aldrich Pvt. Ltd., Germany. All the chemicals were used as received without any further purification. Ultrapure water of pH 6.4–6.5 (Sartorius, Germany) was double distilled again and used for all the experiments, the reagents were filtered with 0.2 l nylon 6, 6 membrane filter paper from Pall Life science, USA. Sodium thiosulphate (pentahydrate) and oxalic acid were used from Rankem (India). Pure slant culture of *Malassezia furfur* (strain 1344) was obtained from Institute of Microbial Technology, Chandigarh, India, and maintained in Emmon's modified medium. Emmons modified media was obtained from Himedia Laboratories Pvt.Ltd. Spectrophotometric ananlysis was done using, UV-3600, Shimadzu, Japan and optical microscope from Hund, Germany was used. Glassware and plasticware like petridishes and culture tubes were obtained from Tarsons, India.

3.2 MEDIA COMPOSITION AND CULTURE CONDITIONS:

Media for growth and maintainance contains:-

Dextrose	40 g/L
Peptone	10 g/L
Agar	18 g/L
Corn oil	2 ml/L).

Growth conditions:- Temperature: 293K

pH: 6.7 optimum, however pH of 5-6 was maintained in our experiments

Incubation time: 7 days

Aerobic growth necessary.

3.3 MEDIA PREPARATION AND CULTURE MAINTAINANCE:

The growth media with the given composition was prepared in conical flasks and autoclaved at 15l psi for 15 min (394 K). Initially cultures were grown in liquid media and when substantial growth was observed, sub culturing was done onto agar plates using glass petri plates (10mm diameter). Growth conditions were maintained as required and a couple of cultures were

stored at freezing temperature using glycerol slants for long term storage. Active cultures were refridgerated. However to observe the lipid dependence of growth for the organism subculturing was done using media where corn oil was replaced by almond oil, coconut oil and butter and observed. The same proportions were used for both liquid and solid cultures.

3.4 SYNTHESIS OF SULPHUR NANOPARTICLES:

Stock sodium thiosulphate was prepared by dissolving solid thiosulphate in double distilled water and the same was done for oxalic acid.

In an acidic solution, sodium thiosulphate undergoes through a disproportionation reaction to sulfur and sulfonic acid according to

$Na_2S_2O_3 + 2H^+ \rightarrow 2Na^+ + SO_2 + S \downarrow + H_2O$

$SO_2 + H_2O \rightarrow H_2SO_3$

After mixing the reactants, 40 min equilibrium time was given for the completion of reaction organic acids. After equilibration, the sample was sonicated in a bath for 2 min and particle size was measured by DLS method immediately. CMC of CTAB was measured by Wilhelmy plate technique with a surface tensiometer (DCAT-11EC, Data Physics, Germany). A constant temperature 28 ± 0.5 ˚C was maintained throughout the experiments.

The sulphur formed as a product of this reaction has the same molarity as thiosulphate added, according to the stoichiometry of the reaction, (since complete reaction is taking place) and hence for future experiments, concentration of nanoparticles of sulphur has been calculated on that basis.

3.5 ANTIFUNGAL ACTIVITY OF SULPHUR NANOPARTICLES ON MALASSEZIA FUFUR CULTURE:

(i) Cell density measurement:

Before conducting antifungal assays, it is necessary to approximately know the number of cells that are being inoculated. This was done with the help of Haemocytometer (Neubauer Haemocytometer, China) method of counting cells. Here a small amount of pure, viable colonies were scratched from a petri plate and mixed thoroughly with 2ml of sterile water to make a homogenous suspension. A small amount of the solution was put onto the haemocytometer, and cells were counted under an optical microscope using 10X objective lense. The cell density

before inoculation was $150x10^4$ cells/ml. This served as a stock solution, from which diluted inoculums were prepared. About 10000, cells/ml was the final cell density used for experiments.

(ii) Inhibition of colonial growth:

This test aims to find a concentration of sulfur nanoparticles of specific size for total inhibition of growth by spread plate method [Zhang and Chen, 2009]. Keeping the size constant, SDA agar plates supplemented with different concentrations from 0.0256 mg/ml to 0.128 mg/ml CTAB stabilized nanoscale sulfur particles (~35-55nm).from a stock of 0.128mg/ml to inspect their fungicidal activity (i.e. 0.2 to 1 fraction dilution of maximum working volume of 500µl, 4mM sulphur solution). Working volume added to the plates was 500µl for both nanoparticles solution and microbial seed solution Active culture was dispersed in sterile water, diluted 40 times (Cell density about 500 cells/ml, though in later experiments, more concentrated cell suspensions were used), and a volume of 0.5 mL from diluted culture used as a seed for the CTAB-Sulfur plate studies, SDA plates without nanoparticles and/or surfactant CTAB were cultured under the same conditions and used as negative control, while all other plates served as positive control. The controlled plates were incubated for 3 days at 30°C, while the plates with sulfur nanoparticles incubated for 5 days (as fungal colonies had delayed growth) and the number of colonies on the plates were counted.

(iii) Growth inhibition tests:

To determine the fungal growth in the presence of sulfur nanoparticles (in CTAB liquid system), isolated fungal species was allowed to grow in the mixture of particles and SD broth (liquid media). Growth was monitored by allowing 2 ml of active fungal culture seed (corresponds to 2×10^7 cells per ml) to grow in liquid SD media (10 ml) modified with sulphur-CTAB solution. Along with all, blanks without CTAB and/or sulfur particles (control) also incubated with culture for 5 days at 30°C. The turbidity of the growth cultures were analyzed by spectrophotometer, and absorbance recorded at 530nm [Ezzi and Lynch, 2005].

Also a comparative study was made using bulk sulphur vs nanosulphur, for the microbial growth characteristics, keeping all other parameters constant. Bulk sulphur solution of same strength, was prepared using, the same reactants, only adding CTAB after reaction is complete, and sulphur precipitate is observed.

CHAPTER 4

OBSERVATIONS AND RESULTS

4. RESULTS AND OBSERVATIONS

4.1 GROWTH OF M. FURFUR:

Malassezia furfur grew as white to tan cream in colour and smooth pasty yeast like appearance over the solid medium and as pale opaque mass in liquid medium. Growth was however immensely dependent on the lipid source provided in media if all other components remain same. In standard corn oil, which is the specified lipid source in literature, the culture grows optimally after 6-7 days. However growth is profuse in coconut oil and butter, the latter taking only 3-4 days of incubation. Curiously the fungus shows growth restriction and hibernation in presence of almond oil. This fact was established by inoculating the cells onto S-D agar with almond oil in which the cells showed initial sporadic growth for 2 days and then no growth at all; however they grew again on sub-culturing onto butter showing that they had not lost their viability.

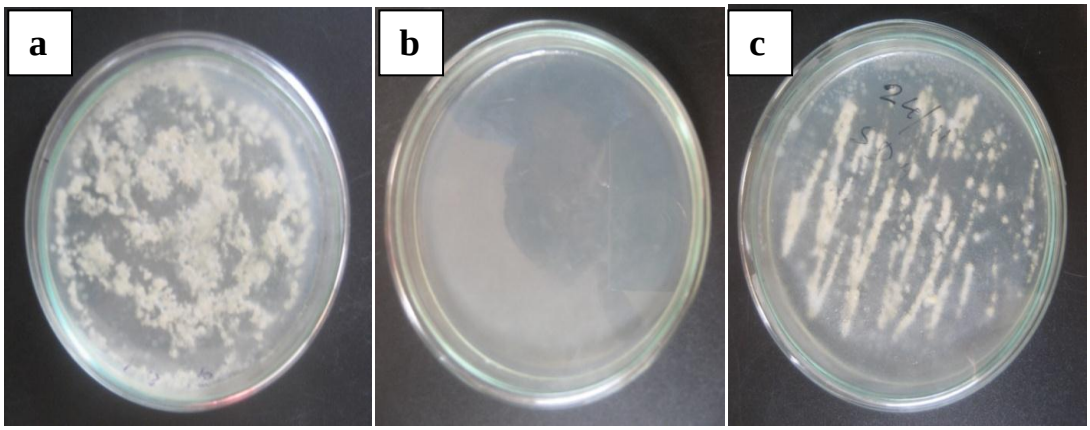


Figure 6: Growth of cells in (a) butter, (b) almond oil, and (c) coconut oil after 6 days incubation.

Hence for further experiments butter would be used as oil source with incubation of 4 days. It is also to be noted that the fungus does not grow at all in absence of a lipid source.

4.2 CHARACTERISTIC MORPHOLOGY OF M.FURFUR:

Detailed microscopic analysis of the cell showed bottle shaped cells with one side round and other flattened, over which a bud like structure grows. Constriction is observed at the intersection.

Very rare hyphae and no spores have been observed.With aging, cells coalesce and show wavy nature; single layered colony formation is observed. This aggregation is better observed with methylene blue staining as shown in figure 7.

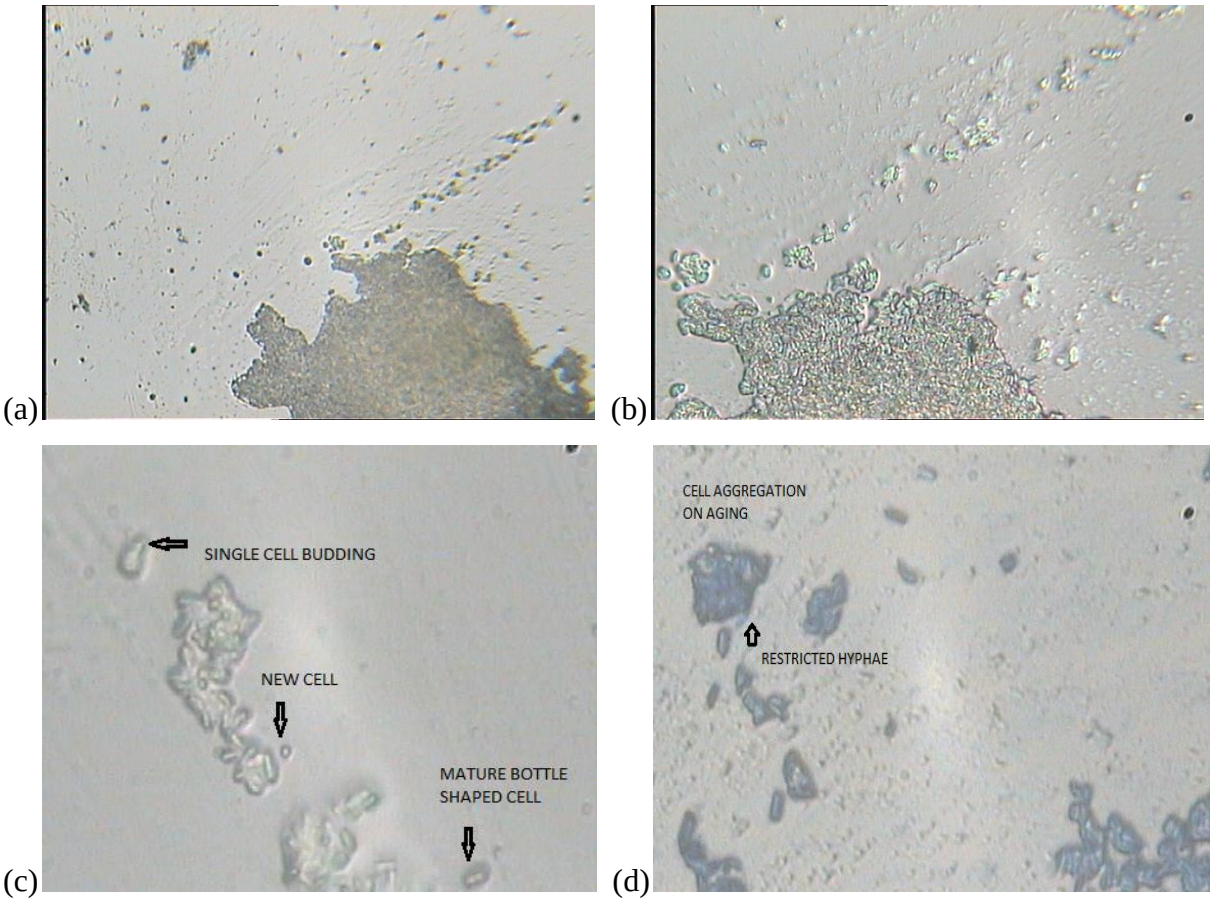


Figure 7: .Microscopic view of M.furfur showing detailed cell morphology (40X optical lens): (a) and (b) showed aggregation and colony structure, (c) and (d) shows identifiable distinct .structures on staining with methylene blue.

4.3 SULPHUR NANOPARTICLE FORMATION IN CTAB:

Sulphur nanoparticles were synthesized by reacting thiosulphate with oxalic acid in 1:6 stoichiometric proportions in pure CTAB solution, with surfactant concentration fixed at a concentration above its CMC value (0.93 mM/L). This makes sure that the number of free surfactant monomers in solution is constant throughout the process and system is stable.

Bulk sulphur could be prepared by the same means, only if CTAB is not present in reaction media. The CTAB was later added to keep uniform concentrations. Bulk sulphur solutions

become pale white and turbid (precipitation observed on standing), while nanosulphur solutions were completely transparent as in figure 8.

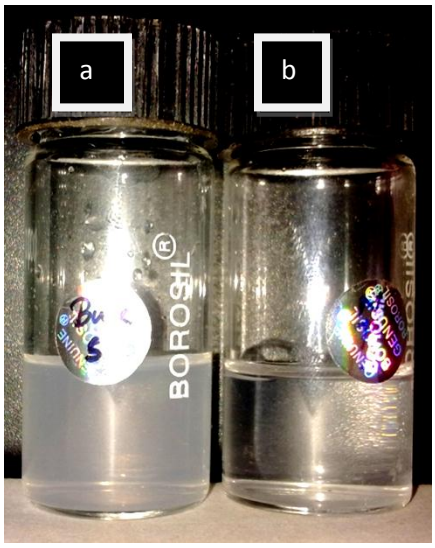


Figure 8: Physical appearance of sulfur suspension, from 4mM thiosulphate solution: (a) Turbid bulk sulphur, (b) Transparent nanosulphur.

The size distribution as obtained from DLS study shows formation of sulphur nanoparticles with mean size range approximately 55nm. As nanoparticle size is strictly dependent on the all the component variations, the reaction medium conditions were kept constant and experiment repeated several times to prove reproducibility. Basically three different thiosulphate concentrations, 4mM, 5mM, 6mM were checked for minimum size. Oxalic acid was added in relative stoichiometric ratio of 1:6 at all times and surfactant concentration remained fixed at 2.7mM. Minimum disturbance was obtained in case of 5mM concentration while a stable mean particle size of 35nm was obtained using 4mM thiosulphate concentration.

The sulphur nanoparticles thus synthesized is appropriate to permeate chitinous cell wall of fungal species and hence can be used as an antifungal agent. Particle concentration distribution is above 5000 i.e. substantial amount of particles has been synthesized. To obtain and utilize the smallest size of 35nm- 55nm of sulphur nanoparticles for the fungicidal assay, 4mM concentration of thiosulphate was used for further experiments.

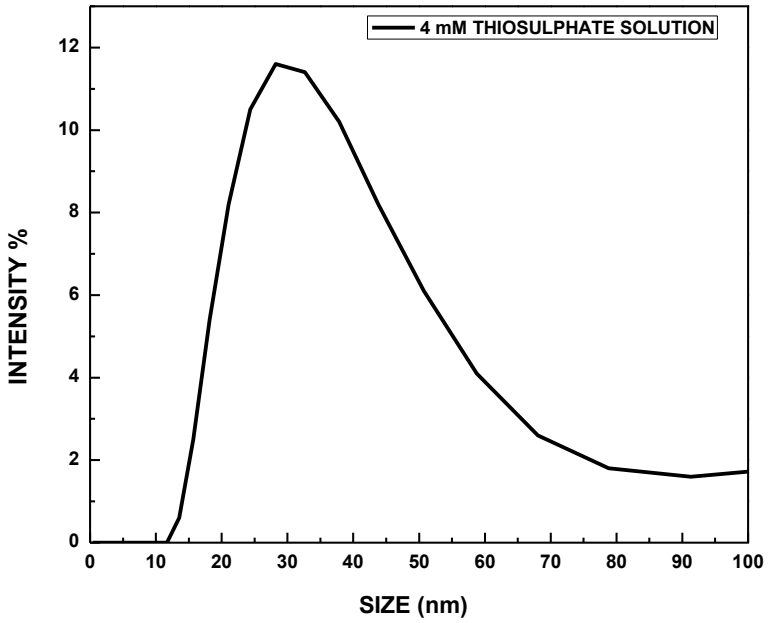


Figure 9a: Intensity vs size distribution curve and particle vs size distribution curve of sulfur nanoparticles formed from 4 mM thiosulphate concentration in CTAB.

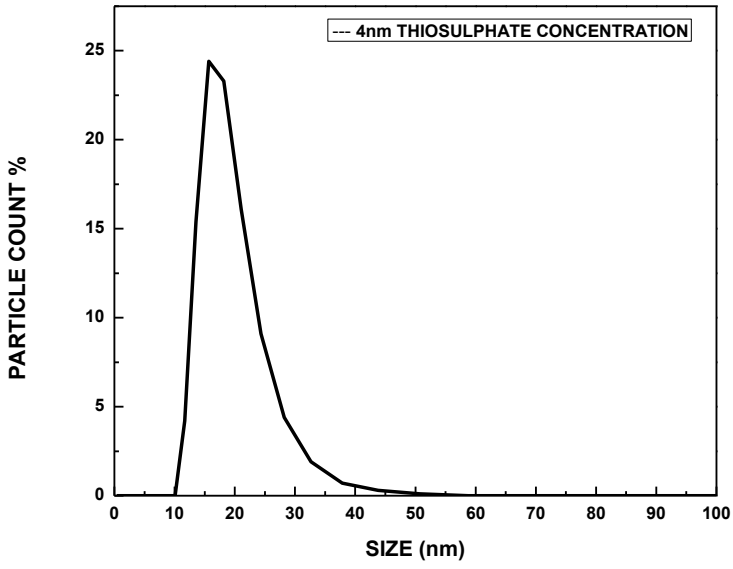


Figure 9b: Particle count vs size distribution curve and particle vs size distribution curve of sulfur nanoparticles formed from 4 mM thiosulphate concentration in CTAB.

4.4 XRD AND SEM ANALYSIS OF NANOPARTICLES

To confirm and see the structural aspects of the nanosulphur formed, an XRD analysis was done of the solution sample. The XRD samples are prepared by successive washing by distilled water, to get pure sulfur peaks .The positions and intensities of the XRD diffraction peaks are same as the literature values for orthorhombic sulfur with S 8 structure [JCPDS PDF number 77-0145]. Sharp peaks showing proper crystalline structure was observed (figure 10).

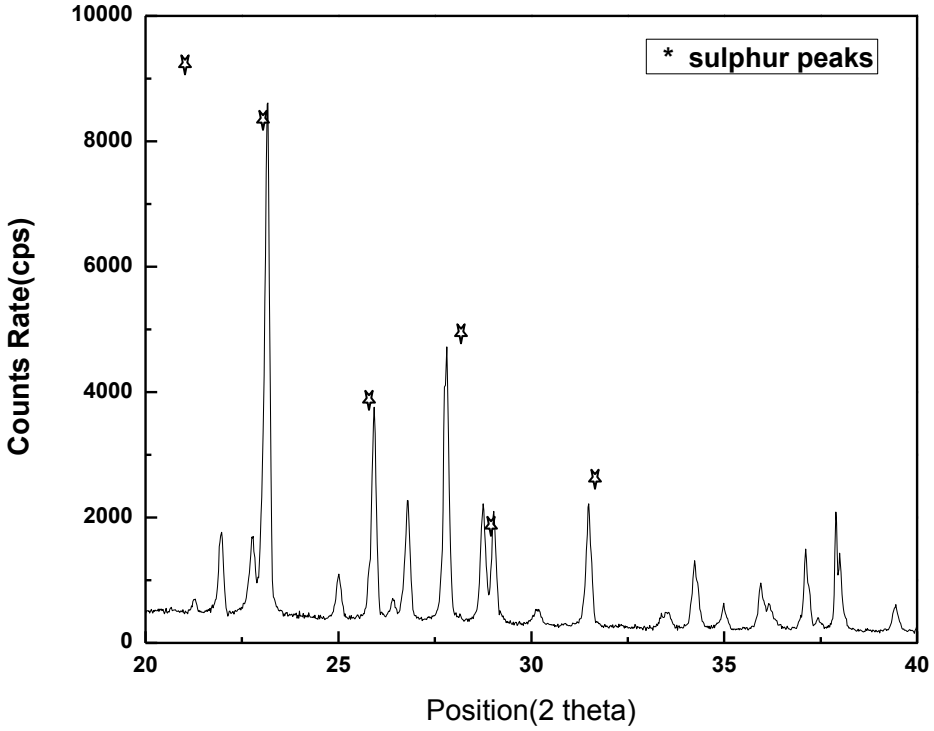


Figure 10: XRD pattern of sulphur particles using oxalic acid and CTAB.

Under Scanning Electron Microscope, the shape and structure of the sulphur particles were viewed. The particles were, spheical in shape, and showed loose aggregation. Closer focusing on the the aggregated structures, showed that each aggregate is a collection of tinier particles, which at around 25000X- 1,000000X, show, solid compact particles of all less than 100nm in size, around 55±10nm on average Though not completely homogenous, the size distribution is uniform, with no huge crystals of bulk sulphur. This data was agreeable with data found in previous literature. EDAX analysis, established the particles as that of sulphur.

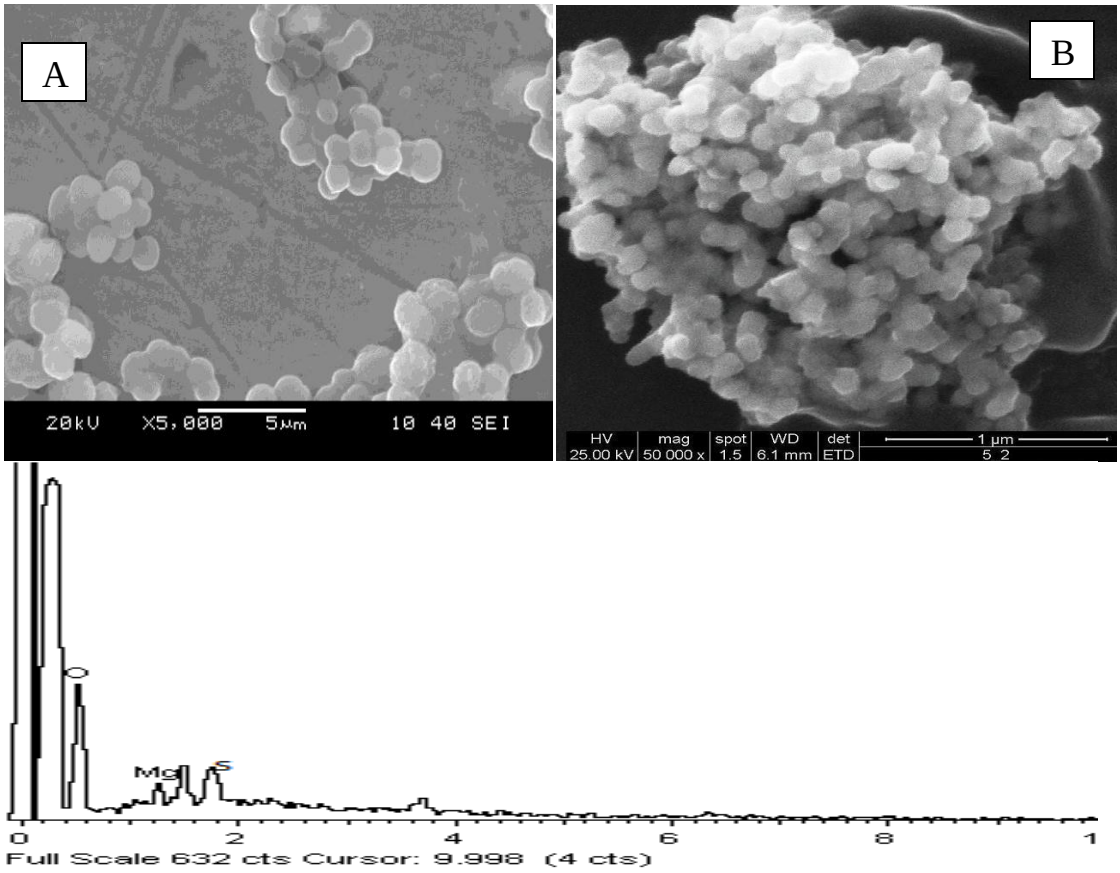


Figure 11: SEM images of nanosulphur obtained using thiosulphate, oxalic acid and CTAB, at different magnifications, (A)5000X ,(B)50000X.Below, EDAX data of the same.

4.5 ANTIFUNGAL ASSAY

(i) Inhibition of colonial growth in solid media:

Fungicidal tests were performed against the isolated *Malassezia* species to know the minimum concentration required for complete inhibition of fungal growth. Since CTAB mediated synthesis of sulfur nanoparticles produce almost uniform particle size of ~40±10nm for a narrow range of reactant concentration, so the effect of particle concentration was studied by simply diluting the mother liquor with CTAB surfactant (so that concentrations of CTAB and other reactants/products remain same, and consequently particle size remain grossly consistent). Figure 12 shows the number of fungal colonies grown on SDA plates as a function of concentration of sulfur nanoparticles. The results show a decrease in number of colonies with an increase in concentration of sulfur nanoparticles (figure 12-13). Inhibition is also evident in plates modified with pure CTAB at 3CMC (i.e. conc. at which it is present in nanoparticles

solution). Therefore the inhibition of fungal growth is a synergistic effect of both nanoparticles and surfactant CTAB. To negate the effect of the later, a reference plate having same fungal inoculations to which thiosulphate-deficient concoction had been added is used as blank and colony inhibition of nanoparticle- modified plates calculated with respect to it. This would negate the effective growth inhibition ability of pure CTAB (and any other factors) on SDA plate and the net inhibition in presence of only sulphur nanoparticles, would be shown individually. A concentration of 0.0512 mg sulfur (in 0.8mL) on SDA plate was able to inhibit ~93% of fungal growth and 0.128 mg (in 1mL) approximately, resulted in complete inhibition of growth, for low inoculation of within 500 initial cells. For higher concentration of inoculate cells (>1000 cells), percentage inhibition by the sulphur nanoparticles is less, though finally at higher than 0.256mg sulphur nanoparticles concentration, the percentage inhibition is high and close to hundred percent.

The probable explanation for this inconsistency can be derived from the mechanism by which nanoparticles interact with microbial cells. Each fungal cell can interact with a finite number of sulphur nanoparticles only, which permeabilize through its cell boundary. Since the number of nanoparticles is also finite and countable for specific solution strength, it can be inferred that a specific concentration of nanoparticles can impregnate a specific number of cells only. If the initial cell inoculates concentration is low, the nanoparticles added are in excess or substantially equal, the percent inhibition curve is almost linear with a steep slope.

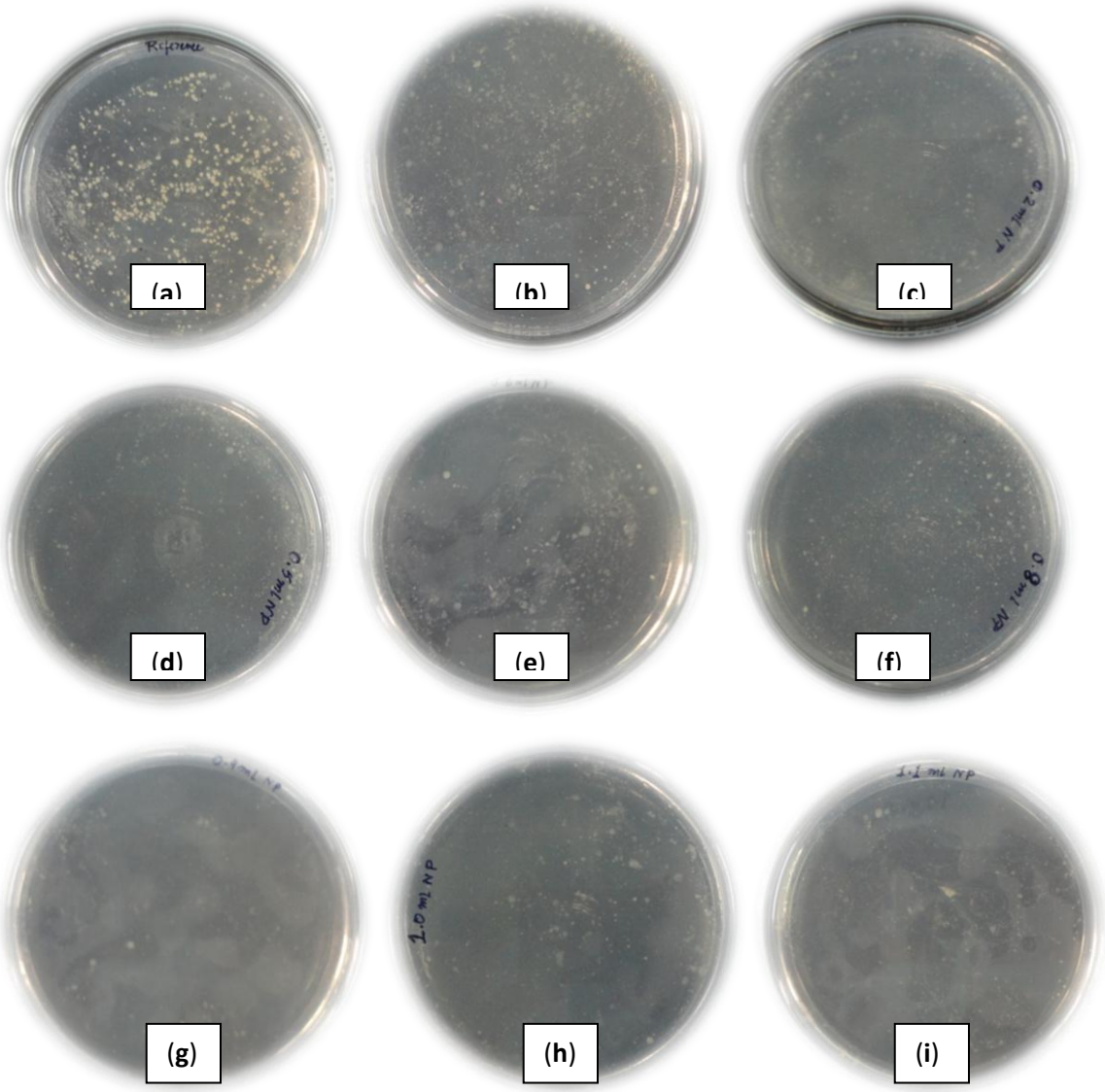
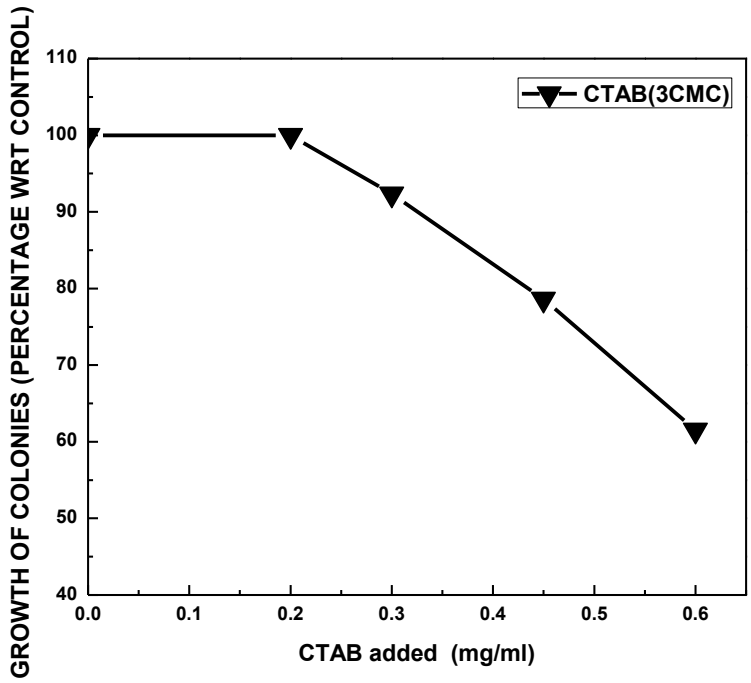
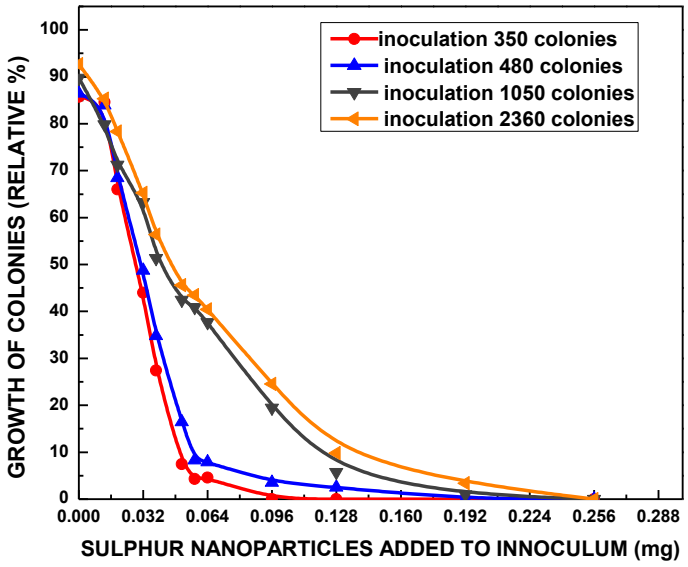


Figure 12: Gradual reduction in growth of colonies on SDA plates for increasing concentration of nanoparticles (a) without particles or negative control, (b) 0.2ml, (c) 0.3ml, (d) 0.5ml, (e) 0.6ml, (f) 0.8ml, (g) 0.9ml, (h) 1ml, (i) 1.5ml of stock 10ml, 4mM/L. Seed culture is 1ml in volume (500 cells/ml).



(a)



(b)

Figure 13: Inhibition of fungal colony in SDA agar plates in presence of (a) pure CTAB (3CMC) and (b) sulphur nanoparticles-CTAB at different initial microbial inoculation (no. of colonies inoculated 350, 480, 1050 and 2360 respectively)

At higher inoculation (>1000), the particles count, though being increased gradually with increasing concentration, cannot match that of cell count and hence percent inhibition is low (though the number of colonies inhibited is more or less constant for all plates, with any inoculation). Only at very high nanoparticles concentration, a sudden break is observed in the percent colony count (where nanoparticles number is higher than cells) and total inhibition is observed only at high sulphur concentration of 0.256 mg.

However it must be noted that, CTAB too plays a good part in inhibiting microbial growth. As we see, impact on yeast inhibition is steadily increasing with concentration. Here, in this experiment, the control plate was inoculated with just microbes with no reagents (sterile water for volume make) and the colony growth percentage was calculated in reference to it. While adding sulphur-CTAB solution to different SDA plates, the concentration of CTAB was maintained at 3CMC, i.e. fixed wt of 0.497mg of CTAB was added to all. This amount of CTAB inhibits approximately 15% of colonies, anyway as intrapolated from figure 13(a), the rest is the work of sulphur nanoparticles. It can be argued that, if CTAB is itself killing such high amount of cells, then what's the worth of nanosulphur's antimicrobial property. In this regard it must be remembered that 3CMC of CTAB (2.7mM/L) is a very high concentration, and almost a necessary wastage in the experiment. Literature shows, that concentration, size and other parameters of the nanosulphur particles, is independent, ideally at any concentration of surfactant above CMC, and practically all fluctuations are removed at 2CMC [Choudhuri and Paria, 2011]. We have maintained a still higher value, to adjust the impact of other changes, like pH, temperature, effect of growth media and so on. So even if the surfactant amount is lowered, which it practically advisable, the nanoparticles concentration remains constistent, and so does its antimicrobial activity (which is the focus of our study).

(ii) Spectrophotometric analysis of liquid broth:

To test for the same in liquid media, fungal inoculate was cultured in liquid media to which sulphur nanoparticles were added (except negative control with only cells and blank control with cells and thiosulphate deficient solution of same strength) and their absorbance measured at 260nm. This was done to confirm presence of live cells. However since at this wavelength, proteins and other components of media, interferes with the data, the growth curve (in this case mortality curve), was plotted by taking absorbance at 530nm. The absorbance

pattern showed decreasing turbidity and consequent decreasing cell count with increasing sulphur nanoparticles concentration with respect to both negative and blank control. A calibration curve of absorbance (at 530nm) vs cell concentration was first plotted with known concentration of cells (using haemocytometer), to infer the cell density of experimental samples.

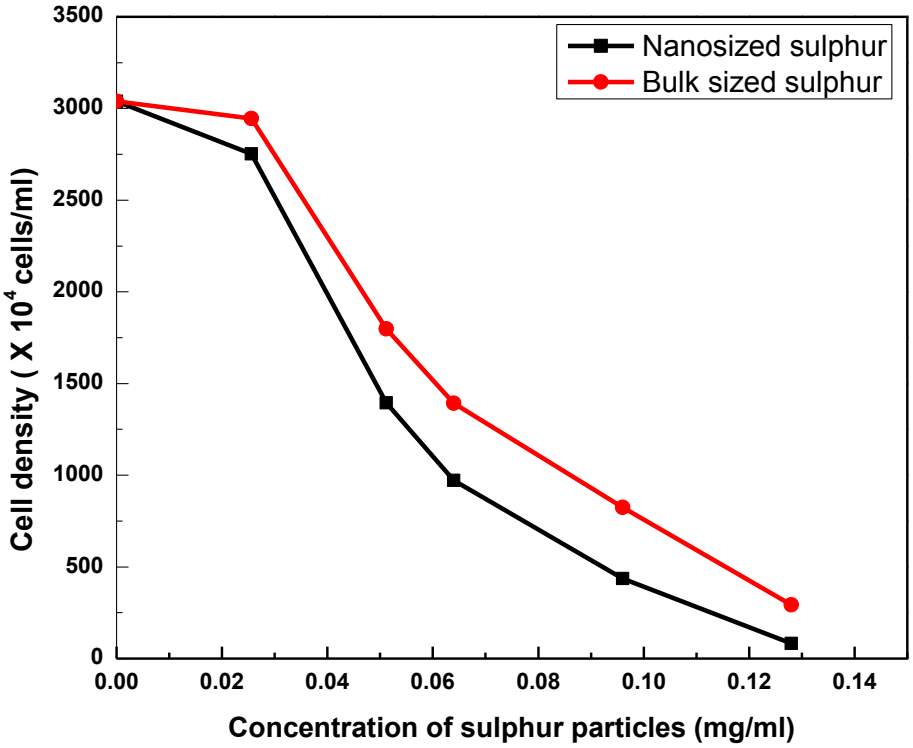


Figure 14: Cell growth in presence of sulphur nanoparticles in comparison to that of bulk particles of same strength.

In the liquid broth study, a comparative analysis was also done, with respect to normal bulk sulphur of the same series of concentrations. This study was not possible in agar plate study, due to precipitation and crystallization of bulk sulphur, making it difficult to uniformly plate. From figure 14, it is noted that the inhibition capacity of nanosulphur is about 20% more than bulk sulphur (size 1000nm-2000nm as mentioned in literature).

The initial inoculation strength here was steady at 10^7 cells/ml with 4 sets of experiments done. The inhibition rate obtained through colony count on agar plates and optical density analysis of

liquid broth both show same rate of inhibition by the nanoparticles system, with approximate error of 5%.

4.6 SEM ANALYSIS OF M. FURFUR:

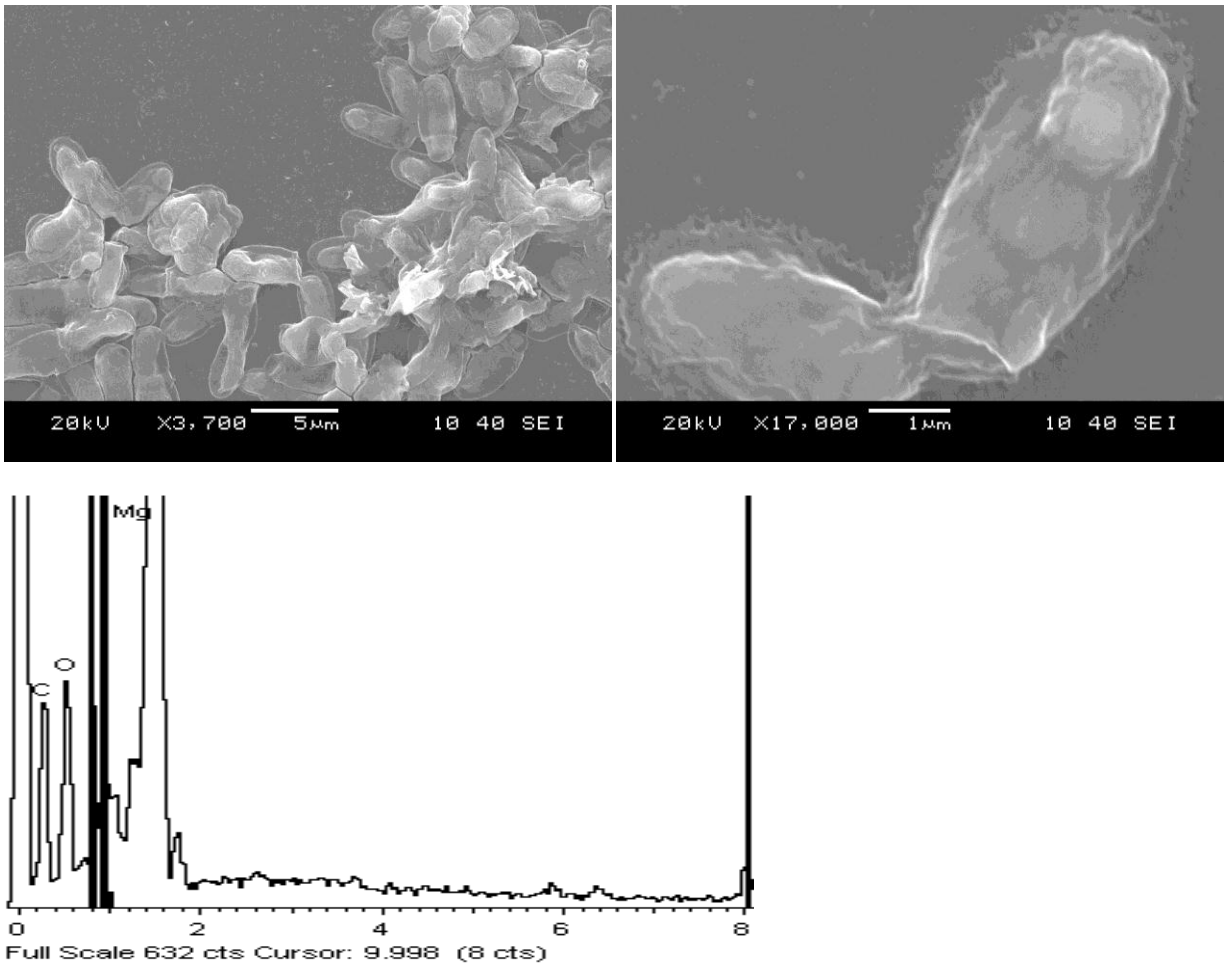


Figure 15: SEM figure of normal *M. furfur* cells, at different magnifications, and the EDAX analysis showing high carbon content.

The figures 15 and 16 show the electron microscope view of *M. furfur* cells, before and after treatment with sulphur nanoparticles. Besides the morphological structures already seen through optical microscopy like, no spore or hyphae and and bowling pin like structures, other notable features include:

- Transparent lipid sheath covering around the cell

- Elongated cells with ‘phialide fitted with collarette’ structure of cell
- Size of single mature cell is around 1µmX10µm.

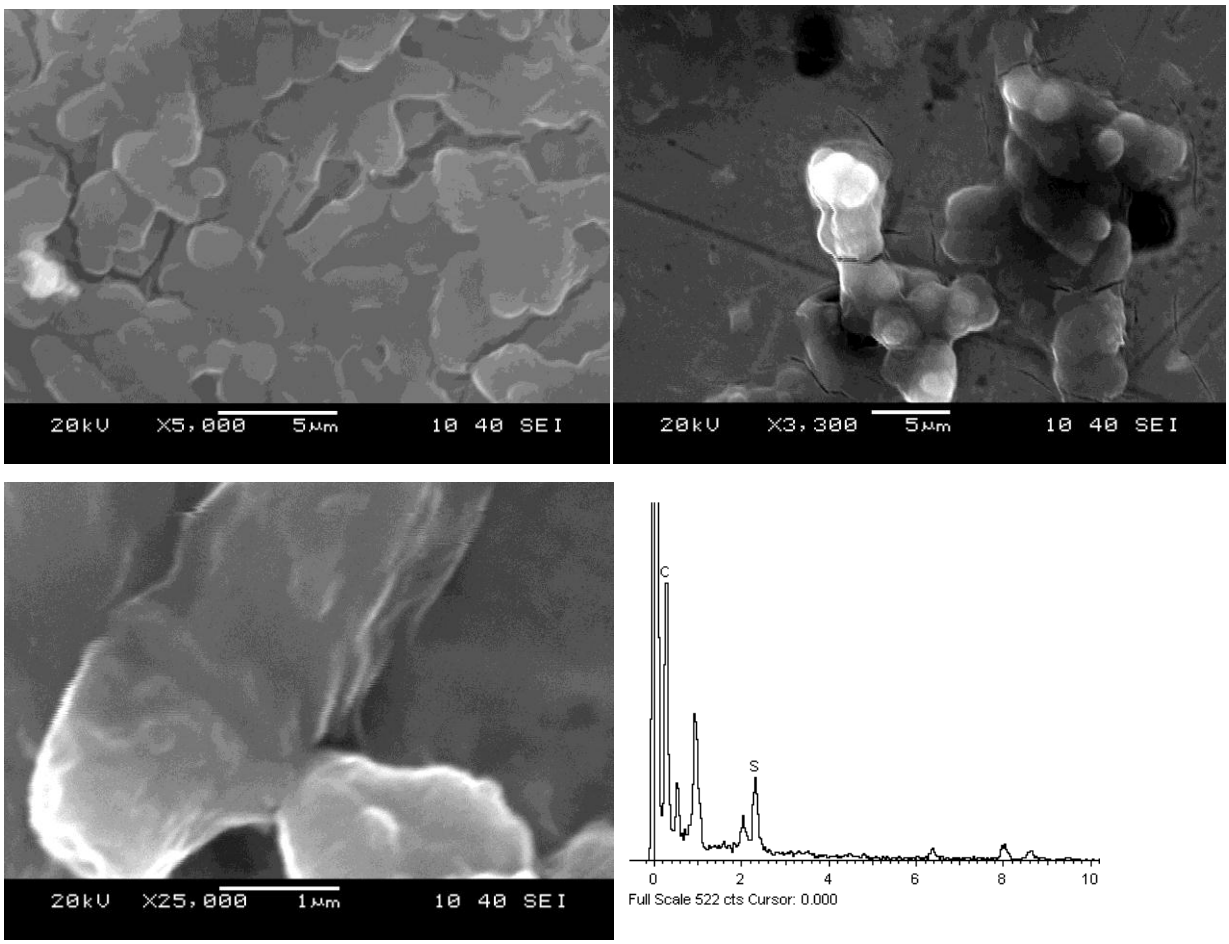


Figure 16: SEM and EDAX of sulphur nanoparticles treated *M.furfur* cells.

After treatment of *M. furfur* cells, with nanosulphur solutions, the cells show certain alterations in structure.

- Firstly, the roughness of the cell surface increases.
- Thinner or no lipid sheath covering the cell.
- The cells appear less elongated, more constricted and almost spherical in structure.

The changes observed after treating the yeast with sulphur nanoparticles under SEM, explains why reduction in colony size and alteration of culture texture from smooth slimy paste to

discontinuous flakes is visibly seen while studying agar plates. The constriction of the lipid sheath is a result of surfactant action, while the cell roughness is a due to increased porosity of the cell boundaries in presence of nanoparticles which infiltrate through the sheath. Also since the cell size is substanstially getting reduced, the same is seen in agar plates, where the colonies become notably diminished in size, when growing in presence of the nanoparticles-surfactant solution.

Thus the yeast inhibition efficiency of the nanoparticles is comparably high and almost equivalent to the values obtained for conventional fungicidal agents.

CHAPTER 6

CONCLUSIONS

5. CONCLUSIONS AND FUTURE PROSPECTS:

We therefore conclude from our experiments, that both surfactant CTAB and sulphur nanoparticles are antimicrobial in nature and their inhibititory action increases with their concentration, for a fixed amount of inoculums.

The fungus M. furfur is growing consistently in the media with healthy growth and no inherent decaying effect. Butter has been the best lipid source showing growth in 4 days which is much less than 7 days growth observed earlier.

Evidently, nanosulphur of 40±10nm, has greater efficiency as the fungicide, over bulk sulphur (1000nm approx.), which is about 20% more in the case of *Malassezia*. Concentration of nanosulphur, corresponding to total inhibition of growth, depends upon initial strength of inoculums.

The close microscopic views, gives us a clear idea of the yeast cell's interaction with nanosulphur-CTAB solution. Gross changes in structure of the viable yeast cells which have been studied under electron microscope, shows the initial attempt of the yeast to adapt to the environment, and condense within.

With all these experiments done, we could very well say that sulphur nanoparticles, thus synthesized in surfactant base, is a good potential antidandruff agent, and the concept could develop a formulation of surfactant and nontoxic sulphur nanoparticles for treatment of dandruff, in future.

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